Input DNA Ratio Determines Copy Number of The 33 kb Factor IX Gene on *De Novo* Human Artificial Chromosomes

Amy M Breman[1], Camie M Steiner[1], Roger B Slee[1] and Brenda R Grimes[1]

[1]*Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, Indiana, USA*

Human artificial chromosomes (ACs) are non-integrating vectors that may be useful for gene therapy. They assemble in cultured cells following transfection of human centromeric α-satellite DNA and segregate efficiently alongside the host genome. In the present study, a 33 kilobase (kb) Factor IX (FIX) gene was incorporated into mitotically stable ACs in human HT1080 lung derived cells using co-transfection of a bacterial artificial chromosome (BAC) harboring synthetic α-satellite DNA and a P1 artificial chromosome(PAC) that spans the FIX locus. ACs were detected in ≥90% of chromosome spreads in 8 of 19 lines expanded from drug resistant colonies. FIX transgene copy number on ACs was determined by input DNA transfection ratios. Furthermore, a low level of FIX transcription was detected from ACs with multiple transgenes but not from those incorporating a single transgene, suggesting that reducing transgene number may limit misexpression. Their potential to segregate cross species was measured by transferring ACs into mouse and hamster cell lines using microcell-mediated chromosome transfer. Lines were obtained where ACs segregated efficiently. The stable segregation of ACs in rodent cells suggests that it should be possible to develop animal models to test the capacity of ACs to rescue FIX deficiency.

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INTRODUCTION

Current gene therapy approaches favor the use of viral vectors due to their capacity to deliver genes into a large population of target cells.[1] However, advances in stem cell technology will permit genetic modification and *ex vivo* expansion of engraftable stem or progenitor cells, opening the way for alternative vector systems. Despite some notable successes with viral vectors, problems have been encountered with immunogenicity, activation of oncogenes and failure to provide sustained transgene expression. A novel vector system that may overcome some of these problems is the human artificial chromosome (AC). Human ACs offer several advantages. (i) They can accommodate megabases (Mbs) of DNA including entire genomic loci with associated regulatory elements, thus maintaining an appropriate response to all regulatory inputs and promoting long-term transgene expression. (ii) They are non-integrating, thus preventing insertional mutagenesis. (iii) They should not elicit an immune response.

Human ACs form *de novo* following introduction of centromeric α-satellite DNA into cultured cells using transfection, invasive bacteria or transduction (reviewed in refs. 2,3). ACs are 1–10 Mb in size, comprising multimers of the input DNA and are maintained alongside the host chromosomes, exploiting the host cell's natural replication and segregation machinery. They assemble a functional kinetochore, the proteinaceous structure that attaches to the mitotic spindle at cell division[4] and exhibit low copy number and high mitotic stability. Recently, large transgenes have been assembled into human ACs by two strategies. (i) Co-transfection of an α-satellite construct with a second construct spanning the gene of interest.[5–7] (ii) Transfection or transduction of *in vitro* synthesized constructs incorporating both α-satellite DNA and a therapeutic gene.[8–12] In all cases, transgene bearing ACs were mitotically stable in human HT1080 cells and where examined, transgene expression was detected in human cells.[5,6,9–12] The goals of the present study were to construct human ACs incorporating the Factor IX (FIX) genomic locus (FIXACs), and investigate parameters influencing transgene copy number and regulation. The ability of FIXACs to segregate cross species was examined as a step towards animal model development. We report efficient incorporation of a 33 kb FIX gene (mutations in which cause hemophilia B) into FIXACs using co-transfection of a bacterial artificial chromosome (BAC) carrying an 86.4 kb synthetic α-satellite insert and a 175 kb P1 artificial chromosome (PAC) spanning the FIX locus, into human HT1080 cells. The molar ratio of input DNA molecules determined FIX transgene copy number and influenced transgene regulation with important therapeutic implications. FIXACs were transferred into rodent cultured cells demonstrating the capacity of FIXACs to segregate cross species and generate an efficient donor for future FIXAC transfer to stem/progenitor cells by microcell-mediated chromosome transfer. Transfer of FIXACs into stem and progenitor cells will permit downstream development of animal models to evaluate the potential of FIXACs to rescue FIX deficiency.

Correspondence: *Brenda R. Grimes, Department of Medical and Molecular Genetics, Indiana University School of Medicine, 975 W. Walnut Street, IB130, Indianapolis, Indiana 46202, USA. E-mail: brgrimes@iupui.edu*

RESULTS

Incorporation of the FIX genomic locus into *de novo* human artificial chromosomes

BAC17 (99 kb) incorporates 86.4 kb of synthetic α-satellite (D17Z1) from human chromosome 17 (**Figure 1a**)[13] and is an efficient substrate for *de novo* human AC formation when introduced as a circle into HT1080 cells.[14–16] PACFIX (175 kb) has a 158 kb genomic insert spanning the 33 kb FIX gene plus 4,077 bp of upstream regulatory sequence sufficient to direct liver specific expression of the FIX gene.[17] In order to generate human ACs incorporating the FIX gene (FIXACs), BAC17 and PACFIX were co-transfected into HT1080 cells using either a 1:1, 2:1, or 20:1 BAC17:PACFIX molar ratio. G418^R colonies were expanded and examined by fluorescence *in situ* hybridization (FISH). FIXACs formed efficiently and assembled both input DNA molecules. In 8 of 19 (42%) cell lines, FIXACs were detected in ≥90% of chromosome spreads within a clonal line (**Figures 1b** and **2a** and **b**). In one additional line, FIXACs formed in 50% of spreads (**Figure 1b**). Control transfections of BAC17 alone resulted in formation of ACs (BAC17-ACs) in greater than 50% of chromosome spreads in 5 of 25 (20%) cell lines. This represents an AC formation rate significantly lower than obtained by co-transfection ($P < 0.025$). The average FIXAC copy number of 3.5/cell is similar to BAC17-AC copy number (**Table 1**).[14–16] While integrated copies of the transfected DNA were observed in lines harboring both FIXACs and BAC17-ACs, the integration frequency was low in 6 of 9 lines that formed a FIXAC, ranging from 0–25% of chromosome spreads (**Figure 1b** and **Table 1**). The TX9C2.6 cell line is a subclone of TX9C2 that has FIXACs in 100% of spreads without detectable integrations (**Figure 1b** and **Table 1**).

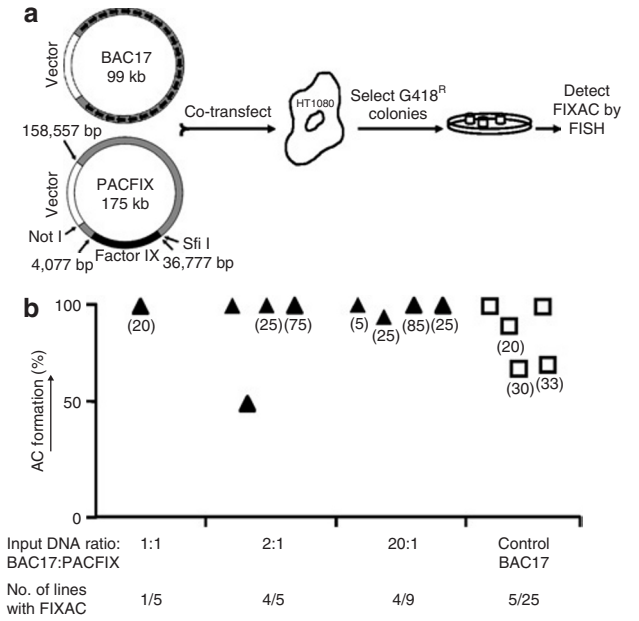


Figure 1 Construction of *de novo* human artificial chromosomes (ACs) incorporating a 33 kb Factor IX (FIX) gene (FIXAC). (**a**) Vectors used to construct FIXAC. BAC17 is 99 kilobase (kb) and has an 86.4 kb insert comprising 32 identical copies of the 2.7 kb higher order repeat, D17Z1, from human chromosome 17. The bacterial artificial chromosome (BAC) vector (VJ104)[13] has a β-geo gene conferring G418 resistance. PACFIX has a sequenced 158 kb genomic insert spanning the 33 kb human FIX gene cloned into the pPAC4 vector. pPAC4 has a Blasticidin S (BS^R) selectable marker gene. A 37 kb *Not*I (*Not*I is present in the vector)/*Sfi*I (position 37090) fragment encompasses the FIX gene (**Figure 3**). The FIX genomic locus was incorporated into *de novo* human ACs (termed FIXACs) following co-transfection of BAC17 and PACFIX into human HT1080 cells. G418^R colonies were expanded and analyzed by fluorescence *in situ* hybridization for the presence of FIXAC (**Figure 2**). (**b**) Human AC formation rates (defined as formation of an AC in ≥50% of spreads). Triangles represent individual cell lines in which FIXACs were detected. Squares represent control cell lines where a human AC formed following transfection of BAC17 as the sole input DNA (BAC17-ACs). The Y axis is the % of chromosome spreads in each cell line which harbored a human AC. Numbers in parenthesis indicate the percentage of spreads in which AC formation was associated with integration of the transfected DNA into a host chromosome. The X axis indicates the molar ratio (1:1, 2:1, or 20:1) of BAC17:PACFIX used in each of the three co-transfection assays, and the number of G418^R cell lines where a FIXAC formed in each transfection. FIXACs were detected in 9/19 lines. FIXACs did not form in the remaining 10 lines. In the control, BAC17 formed BAC17-ACs in 5/25 cell lines. A 6th cell line formed a BAC17-AC in 20% of spreads (data not shown). In the remaining BAC17 control lines, the transfected DNA did not form an AC.

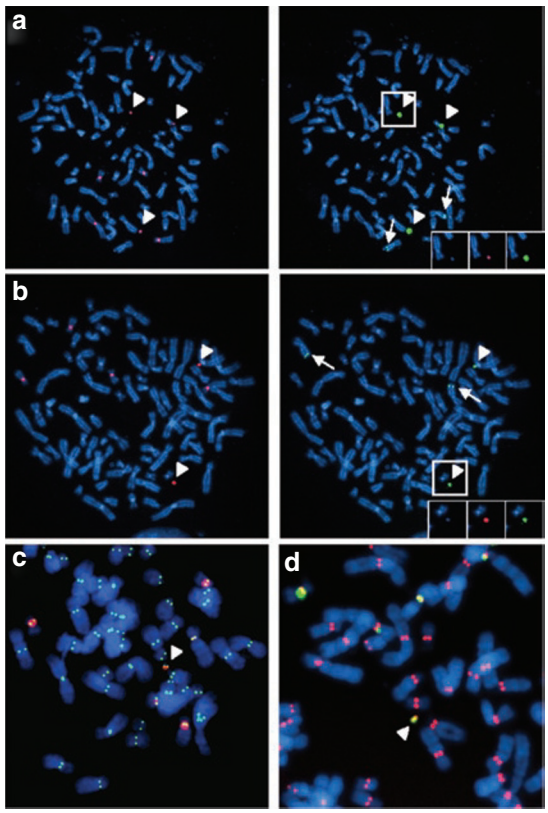


Figure 2 Fluorescence *in situ* hybridization (FISH) analysis and CENP-A detection on FIXACs. (**a**, **b**) FISH analysis of metaphase spreads from TX9A2 (**a**) and TX10B1 (**b**) hybridized with a D17Z1 probe (red, left panel) and PACFIX (green, right panel). FIXACs are indicated by arrowheads. Split images of the same metaphase spread are shown in each panel in (**a**) and (**b**). (**a**, **b**) Left panels: D17Z1 hybridized with FIXAC and endogenous D17Z1 sequences. (**a**, **b**) Right panels: PACFIX hybridized with FIXACs and the endogenous sequences on the X chromosome (arrows). White boxed area is magnified in the inset. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (blue). (**c**, **d**) Chromosomes from cell lines TX9A2 (**c**) and TX10B7 (**d**) were analyzed by indirect immunofluorescence using an anti CENP-A antibody (green signals in **c**, red signals in **d**. FISH analysis using a D17Z1 probe (red in **c**, green in **d** identified FIXAC (arrowhead) and the endogenous chromosome 17s.

Table 1 Properties of human artificial chromosomes harboring a Factor IX gene (FIXAC) in human HT1080 cells[a]

Cell line	BAC17: PACFIX Input ratio[b]	FIXAC frequency (%)[c]	FIX transgene number[d]	FIXAC copy number[e]	Mitotic stability 30 d/ loss rate[f]	CENP-A[g]	FIX mRNA[h]	*de novo*[i]
TX9A2	2:1	100	14.9	3.4 (2–11)	100 (0)	+	+	Yes
TX9C2	2:1	100 (25)	nd	2.9 (1–6)	100 (0)	nd	+	nd
TX9C2.6	2:1	100	25	4.0 (2–5)	100 (0)	+	+	Yes
TX10B1	20:1	95 (5)	0	2.0 (1–4)	95 (0)	+	-	Yes
TX10B7	20:1	90 (25)	0.7	4.8 (1–10)	75 (0.0076)	+	-	Yes

Abbreviations: nd, not done; RT-PCR, reverse transcriptase polymerase chain reaction.
[a]FIXAC is a human artificial chromosome (ACs) that has incorporated a 33 kb human Factor IX (FIX) gene(s) and chromosome 17 α-satellite DNA sequences. [b]The molar ratio of BAC17:PACFIX DNA used in the transfection mixture is indicated. [c]% of chromosome spreads harboring FIXACs within each cell line is indicated. Numbers in parentheses indicate the % of spreads where a signal on a host chromosome was detected in addition to FIXAC. [d]Average FIX transgene copy number/FIXAC was determined by semi-quantitative real-time PCR (**Figure 3d**). [e]Mean FIXAC copy number/cell and range is indicated. [f]% spreads retaining FIXACs following 30 days culture without selection. Loss rate (*R*) was calculated as described in **Figure 3**. [g]Kinetochore formation on FIXACs was evaluated using CENP-A antibody (**Figure 2d** and **e**). [h]RT-PCR and Northern analysis were used to determine whether FIX transcription from FIXAC was detectable (**Figure 4**). [i]FIXACs were considered *de novo* in composition if they did not hybridize to a pan-centromere probe in which D17Z1 sequences were competed out (data not shown).

Five cell lines containing FIXACs in >90% of chromosome spreads were selected for further analysis (Table 1).

Mitotic stability and CENP-A deposition on FIXACs

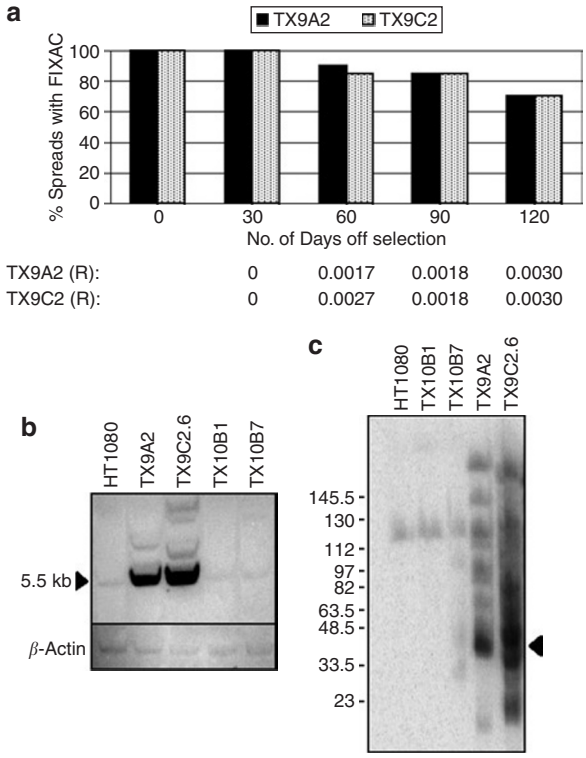
The mitotic stability of FIXACs in HT1080 cells was examined in five cell lines (Figure 3a and Table 1). FIXACs were retained in 70% of cells after 120 days of non-selective culture in lines TX9A2 and TX9C2 (loss rate (R) = 0.003), indicating that FIXACs have assembled a functional centromere and are highly mitotically stable. In three further lines, FIXACs were detected in 75–100% of cells after 30 days culture in the absence of selection, corresponding to loss rates of 0–0.0076 (Table 1). Consistent with the formation of a kinetochore, the centromere-specific histone H3 variant centromere protein A (CENP-A)[18] assembled on FIXACs (Figure 2c and d and Table 1). To rule out the possibility that FIXACs had achieved mitotic stability through acquisition of host α-satellite sequences, chromosome spreads were hybridized with an α-satellite consensus probe that had been preannealed with unlabeled D17Z1 sequences. No signal (above background) was detectable on FIXACs (Table 1; data not shown). This result, together with the complete overlap of D17Z1 and PACFIX hybridization signals with 4′,6-diamidino-2-phenylindole fluorescence, suggests that FIXACs are *de novo* in composition and comprise input DNA sequences only.

Input BAC17:PACFIX ratio determines FIX transgene copy number on FIXACs

The FISH signal intensity with a PACFIX probe was markedly elevated compared to the endogenous X chromosome signal, on all FIXACs generated from the 2:1 or 1:1 input DNA ratios (Figure 2a). In contrast, the FISH signal intensity on FIXACs in 4 of 5 lines generated from a 20:1 input DNA ratio, was similar to the endogenous signal (Figure 2b). These observations suggested that the BAC17:PACFIX input ratio may determine FIX transgene copy number on FIXACs. To examine this possibility further, Southern analysis and semi-quantitative real time polymerase chain reaction (PCR) (qPCR) assays were performed. Genomic DNA was isolated from cell lines TX9A2 and TX9C2.6 (generated from the 2:1 input ratio) and lines TX10B1 and TX10B7 (generated from the 20:1 input ratio). In each sample, the predicted 5.5 kb *Eco*RI fragment was detected by Southern analysis (Figure 3b). Additional weaker signals were also detected in lines TX9A2 and TX9C2.6, indicating a degree of input DNA rearrangement. In comparison to TX10B1 and TX10B7, the 5.5 kb FIX signal was significantly elevated in TX9A2 and TX9C2.6, consistent with a higher transgene copy number per FIXAC in these lines. Based on the qPCR analysis, FIXACs in lines TX9A2 and TX9C2.6 assembled an average of 14.9 and 25.1 FIX transgenes, respectively, whereas FIXACs in lines TX10B1 and TX10B7 assembled either no transgenes or an average 0.7 FIX genes/FIXAC, respectively (Figure 3d). These values are in close agreement with densitometric analysis of the 5.5 kb FIX band detected by Southern hybridization (Figure 3b; data not shown). To assess FIX gene integrity, *Sfi*I and *Not*I digested genomic DNA was examined by Southern analysis (Figure 3c). A strongly hybridizing band at 37 kb, derived from the PACFIX input DNA (Figure 1a), was detected in lines TX9A2 and TX9C2.6 indicating the presence of multiple intact FIX transgenes. In TX10B7, a band at 37 kb was detectable at lower intensity, consistent with the presence of an intact FIX transgene (see **Supplementary Figure S1**). The presence of additional bands indicated that rearranged FIX genes are also present in these three cell lines. In line TX10B1, FIX transgenes were not detected suggesting that only remnants of PACFIX excluding the FIX gene are present on the AC.

Low levels of FIX transcription from FIXACs assembling multiple FIX transgenes

FIX expression would not be predicted in the lung derived HT1080 cell line as FIX is limited in expression to hepatocytes. However, previous reports of transcription of the β-globin transgene on a *de novo* human AC in HT1080 cells[10] led us to examine FIX transcription from FIXAC. FIX transcripts were not detected from the endogenous HT1080 FIX locus, as expected, or in cell lines TX10B1 or TX10B7 harboring low FIX transgene (≤1) copy number FIXACs (Figure 4a and b). In contrast, FIX messenger RNA (mRNA) was detected at a level comparable to the human liver control in lines TX9A2 and TX9C2.6 (Figure 4b and c) that harbor multiple FIX transgenes. Northern analysis confirmed the presence of the predicted 2.8 kb FIX mRNA in both cell lines (Figure 4b). Structural integrity of FIX mRNA in and TX9A2 and TX9C2.6 was further confirmed by reverse



d qPCR analysis

Cell line	Total No. of FIX genes/cell	No. of FIX transgenes/ FIXAC	Average FIXAC copy no./cell
HT1080	2	0	0
TX9A2	52.6	14.9	3.4
TX9C2.6	102.3	25.1	4
TX10B1	1.9	0	2
TX10B7	5.4	0.7	4.8

Figure 3 Factor IX artificial chromosome (FIXAC) mitotic stability and structural analysis. (**a**) Lines TX9A2 and TX9C2 were passaged for 120 days under non-selective conditions. FIXAC retention was analyzed at 30 day intervals using fluorescence *in situ* hybridization analysis with PACFIX and D17Z1 probes. Loss rate (*R*) was calculated using the formula $N_n = N_0 \times (1\text{-}R)^n$, where N_0 = percentage of metaphase spreads with FIXACs in cells cultured under selection, N_n = percentage of FIXAC-containing metaphase spreads after *n* days of culture in the absence of selection. (**b**) Genomic DNA was digested with *Eco*R1, blotted and hybridized with a FIX probe that recognizes a 5.5 kb fragment (upper panel). A β-actin probe was used as a loading control (bottom panel). (**c**) Identification of intact FIX transgenes on FIXAC. High molecular weight DNA was digested with *Not*I and *Sfi*1 to excise a 37 kb fragment (arrowhead) encompassing the 33 kb FIX locus that is specific to FIXAC (**Figure 1a**). In HT1080 cells, the endogenous signal at ~120 kb reflects the presence of two FIX genes in male HT1080 cells that are close to tetraploid in chromosome composition. The positions of DNA size markers are indicated in kilobases (kb). (**d**) Real-time polymerase chain reaction (real-time PCR) analysis (qPCR) on genomic DNA was used to quantify FIX transgene copy number in each cell line. The number of FIX transgenes per FIXAC was determined by dividing the total number of FIX genes (corrected for the two endogenous FIX genes) by the average number of FIXACs per cell. Each sample was normalized to β-actin.

transcriptase PCR (RT-PCR) using primers spanning the FIX complementary DNA (cDNA) which yielded a pattern comparable to the liver control, with little or no evidence of aberrant FIX mRNA transcripts (**Supplementary Figure S2**).

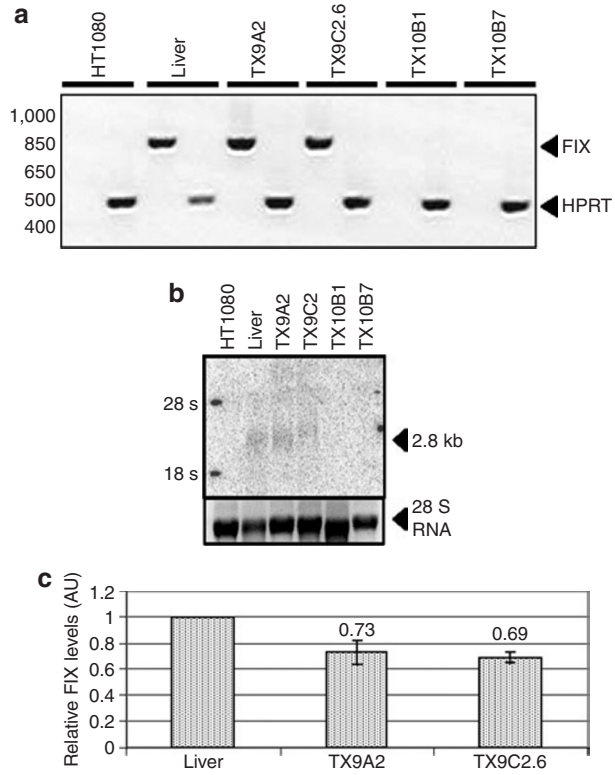


Figure 4 Factor IX (FIX) transcription from FIXACs. (**a**) Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine whether FIX messenger RNA (mRNA) is transcribed from FIXACs. FIX primers amplified a 780 bp product (FIX) in lines TX9A2 and TX9C2.6, as well as the human liver control. In lines TX10B1, TX10B7, and parental HT1080 cells, the FIX RT-PCR product was not detected. Control primers amplified a 498 bp hypoxanthine–guanine phosphoribosyltransferase (HPRT) product. (**b**) Northern analysis. The 2.8 kb FIX mRNA was detected in lines TX9A2 and TX9C2 and human liver, but not in lines TX10B1, TX10B7, or HT1080. The ethidium bromide stained 28S ribosomal subunit is shown as a loading control. (**c**) FIX mRNA quantitation using semi-quantitative real-time RT-PCR. Y axis is relative FIX mRNA levels in arbitrary units (AU). Samples were normalized to β-actin. In HT1080, TX10B1, and TX10B7, FIX mRNA was not detectable (data not shown). Error bars show mean + SD and are based on two replicates.

Stable segregation of FIXAC in rodent cells

A prerequisite for development of a mouse model of AC mediated gene therapy is that FIXACs segregate stably in a rodent cell background. Using the procedure of microcell-mediated chromosome transfer, FIXACs were transferred from TX9C2.6 (**Table 1**) into LA9 (mouse fibroblast) and CHL (Chinese hamster lung) cells. In addition to providing a test of FIXAC segregation potential, LA9 and CHL FIXAC monochromosomal hybrids will serve as efficient microcell donors for subsequent FIXAC transfer into stem cells. Approximately 120 colonies were obtained following fusion of microcells prepared from the TX9C2.6 cell line and CHL cells. FISH analysis of 10 BS^R CHL lines using a human Cot1 DNA probe that hybridizes to all human chromosomes, demonstrated that 9 of 10 lines were monochromosomal hybrids that harbored FIXAC as the only human chromosome (data not shown). Three monochromosomal hybrid lines (CHL10, CHL13, CHL15) in which FIXACs were detected in ≥95% of chromosome spreads without detectable translocations of FIXAC onto host chromosomes, were further

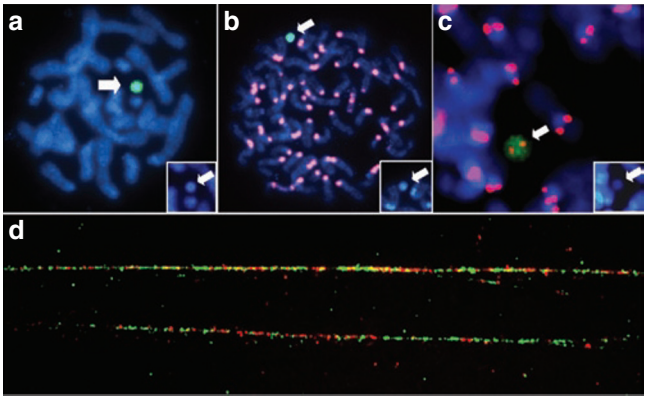


Figure 5 Factor IX artificial chromosome (FIXAC) transfer into rodent cell lines using microcell-mediated chromosome transfer (MMCT). The human HT1080 line TX9C2.6 formed microcells very efficiently and was used as a donor for transfer of FIXAC into rodent cells using MMCT. (**a, b**) PACFIX probe (green) hybridized with FIXAC (arrow) in Chinese hamster lung (CHL) monochromosomal hybrid CHL10 (**a**) and mouse LA9 monochromosomal hybrid L1 (**b**). (**b**) Mouse minor satellite probe (red) is detected exclusively on the endogenous mouse centromeres. (**c**) The mouse centromere protein, Cenp-c, is detected on FIXAC in L1 cells as well as endogenous mouse centromeres. The PACFIX probe (green) confirmed FIXAC identity. Insets (**a–c**) show 4′,6-diamidino-2-phenylindole stained FIXACs. (**d**) Stretched DNA fibers from L1 cells hybridized with a D17Z1 probe (red) and PACFIX (green). The interspersed hybridization pattern suggests that FIXACs are composed of multiple copies of each input DNA molecule arranged as alternating segments.

examined (**Figure 5a**). The average FIXAC copy number per cell was 1.9, 1.1, and 3.4/cell in CHL10, CHL13 and CHL15, respectively. In line CHL15, FIXACs exhibited a high level of mitotic stability and were retained in 90% of cells after 60 days growth without selection ($R = 0.0018$) (**Figure 6a**). FIXACs in lines CHL10 and CHL13 exhibited either a moderate ($R = 0.0114$) or high loss rate ($R = 0.0503$), respectively, demonstrating variation in the segregation efficiency of FIXAC in CHL cells. Four BS^R LA9 colonies were obtained following microcell-mediated chromosome transfer. In 3/4 lines, FIXACs were detected as a high copy number chromosome. Consistent with earlier studies,[19,20] the high copy number FIXACs were mitotically unstable and not further studied (data not shown). In a fourth line, L1, FIXACs were detected in 90% of chromosome spreads at a low copy number (average 1.7/cell), and without detectable FIXAC translocations (**Figure 5b**). Although the mouse kinetochore protein, Cenp-c assembled on FIXAC in L1 cells (**Figure 5c**), FIXACs were retained in only 8% of cells following 30 days in non-selective conditions ($R = 0.0744$). This aggregate loss rate may reflect a uniformly high level of FIXAC instability or FIXAC stability may be heterogeneous such that a subpopulation of cells harbors a mitotically stable FIXAC. To investigate this latter possibility, L1 cells were plated at low density to generate single colonies that were expanded to form subclonal lines. Five subclonal L1 lines were identified in which FIXACs were present in 92–100% of chromosome spreads. Over 30 days passage without selection, FIXAC mitotic stability was higher than in the parent L1 line, with retention of FIXACs in 72–28% of cells ($R = 0.0081–0.0402$) (**Figure 6b**). This improved mitotic stability did not result from acquisition of minor satellite DNA (the functional mouse centromeric sequence) as a minor satellite probe did not hybridize to FIXAC in L1 cells (**Figure 5b**) or in subclonal lines (data not shown). To rule out gross structural FIXAC instability, FIXAC structure was examined by Southern analysis using a chromosome 17 α-satellite probe (**Figure 6c**). The BAC17 construct would produce a 99 kb *Apa*I fragment if no rearrangement of the input DNA had occurred. Fragments ranging from ~10 to 194 kb were detected, confirming input DNA rearrangement during chromosome formation (**Figure 6c**). Furthermore, an identical pattern of hybridization was obtained in all lanes suggesting that FIXACs are structurally stable. Confirmation that FIXACs comprise alternating DNA segments derived from the two input molecules was obtained by performing FISH on L1 extended DNA fibers (**Figure 5d**). Both LA9 and CHL cells tested negative for FIX expression with RT-PCR. However, consistent with the results in TX9C2.6, FIX transgene transcription persisted from FIXACs in rodent cells (**Figure 6d** and **e**). Interestingly, the level of FIX transcription was variable among the rodent cell lines and appeared unrelated to FIXAC mitotic stability.

DISCUSSION

The present study demonstrates that a 33 kb FIX gene can be assembled efficiently into a *de novo* human AC (FIXAC) by co-transfection of two large DNA molecules (PACFIX and BAC17) of defined sequence. The 175 kb human genomic PAC (PACFIX) spans the FIX locus while the 99 kb centromeric BAC17 vector has 86.4 kb of synthetic α-satellite DNA. The AC formation rate was higher using co-transfection than using BAC17 as the sole input molecule (**Figure 1b**). The increased AC formation rate may reflect the presence of additional replication origins, insulator elements for setting up chromatin domains or assembly of transcription factors as a result of incorporation of the FIX genomic segment. In comparison, co-transfection of a hypoxanthine–guanine phosphoribosyltransferase PAC and a chromosome 21 α-satellite PAC into HT1080 cells yielded a lower efficiency with only 5 of 25 cell lines forming an AC in 10–50% of spreads.[5] While differences in vector and α-satellite sequence may contribute to these differences in AC formation rates, it has recently been shown that the genomic segment incorporated into a human AC can also significantly impact this process.[10] We have demonstrated that the ~45 kb polycystic kidney disease 1 locus can be incorporated into ACs (using the co-transfection strategy) with efficiency similar to the FIX locus, and that input DNA ratio in the transfection mixture again influenced transgene number on human ACs (A.M.B., R.L. Bacallao, B.R.G., unpublished results). Interestingly, integration frequencies were markedly lower in this instance. A better understanding of parameters affecting chromosome formation versus integration and increased control over input DNA rearrangements during chromosome formation will be required before *de novo* human ACs can be applied in a therapeutic setting.

In addition to providing correct spatial and temporal regulation, an AC borne therapeutic transgene should be expressed at a physiologically appropriate level, making transgene copy number an important consideration. In the present study we demonstrate that the input ratio of BAC17:PACFIX determined FIX transgene copy number on FIXACs (**Figure 3**). When the BAC17 construct was introduced in 20-fold excess to PACFIX, FIXACs that assembled ≤1 FIX transgene were generated. In contrast, FIXACs generated from a 2:1 BAC17:PACFIX input ratio

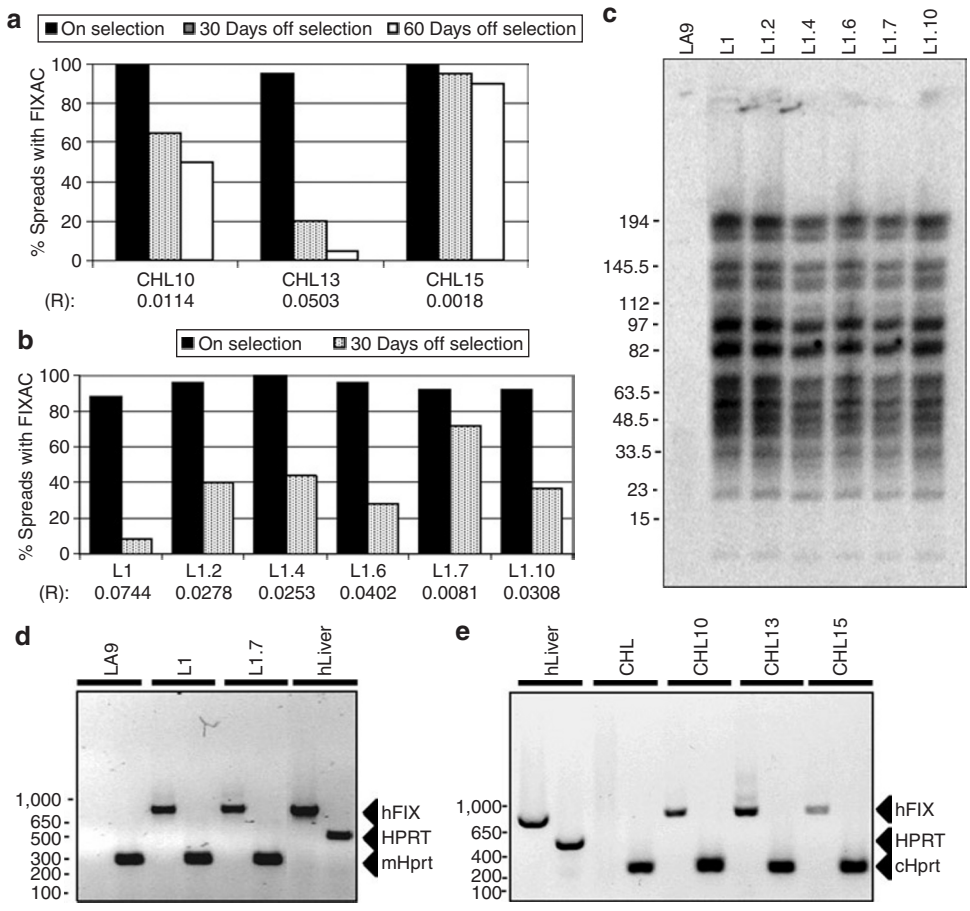


Figure 6 Factor IX artificial chromosome (FIXAC) mitotic stability and FIX transcription in Chinese hamster lung (CHL) and LA9 monochromosomal hybrids. (**a**, **b**) CHL monochromosomal hybrid (MCH) cell lines CHL10, CHL13 and CHL15 (**a**) and LA9 MCH cell lines, L1 and L1 subclones (L1.2, L1.4, L1.6, L1.7 and L1.10) (**b**) were passaged in the absence of selection. FIXAC mitotic stability was measured using fluorescence *in situ* hybridization analysis at 30 and 60 days for CHL MCH lines (**a**) and at 30 days for LA9 MCH lines (**b**). PACFIX was used to detect FIXACs in CHL and LA9 MCHs. In LA9 lines, a mouse minor satellite probe was also used which hybridized only to mouse centromeres and not to FIXACs in L1 and L1 subclones (**Figure 5b**; data not shown). (**c**) Structural integrity of FIXACs in L1 cells and L1 subclones was examined using Southern analysis of *Apa*I digested high molecular weight DNA. Digested DNA was separated on a pulsed field gel, blotted and hybridized with a chromosome 17 specific α-satellite probe, which hybridized exclusively to FIXACs. Positions of mid range DNA size markers in kb are indicated. (**d**, **e**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of FIX mRNA transcription from FIXACs in L1, L1.7 (**d**) and CHL MCH lines (**e**). A 780 bp human FIX RT-PCR product was amplified in L1, L1.7, CHL10, CHL13, and CHL15 complementary DNA (cDNA), as well as in human liver control cDNA. Reactions with mouse Hprt (mHprt, 286 bp product) or human HPRT (498 bp product) primers were included as controls in **d**. Primers spanning either Chinese hamster Hprt (cHprt) or human HPRT (hHPRT) were used as controls in **e**.

incorporated an average of 14.9 and 25.1 FIX transgenes in two independent lines. Furthermore, an association was observed between elevated transgene copy number and a low level of FIX misexpression in lung derived HT1080 cells (**Figure 4**) although previous studies indicated that sufficient regulatory sequences to direct liver specific expression should be contained within the PACFIX construct.[17] FIX mRNA detection in lines harboring FIXACs with multiple transgenes could also indicate that transgenes are not completely silenced. In contrast, no transcription was detected from the single FIX transgene in line TX10B7. Whilst this raises the possibility that copy number is critical to appropriate transgene regulation, inactivation of this transgene through minor rearrangement undetected by Southern analysis, cannot be excluded.

In transgenic mouse models, it has been demonstrated that genes introduced either as naked DNA or on human chromosomes or their derivatives, undergo chromatinization during the differentiation process to result in the correct gene expression pattern (reviewed in refs. 21,22). An important question for their therapeutic application is whether an AC borne transgene introduced via an undifferentiated stem cell from a committed donor is appropriately remodeled. Pertinent data are currently sparse. Mouse studies will allow a comprehensive analysis of the regulation of the AC borne FIX gene and its capacity to provide phenotypic rescue of hemophilia B.

In order to develop a mouse model of AC mediated gene therapy it is necessary that the transgene bearing AC segregates stably in a rodent cell background. Human chromosomes estimated to be less than 50 Mb in size, including *de novo* human ACs, segregated with a reduced efficiency in mouse LA9 cells compared to human cells in previous studies[6,19,20,23] and two reports have implicated chromosome size as a factor in decreased

segregation competence.[15,20] FIXAC assembled the mouse kinetochore protein, Cenp-c, upon transfer into LA9 cells but exhibited a high initial missegregation rate. However, FIXACs exhibited higher mitotic stability in subclonal lines suggesting an improved segregation competence of a subset of FIXACs (**Figure 6b**). Similar results were obtained with a 7–10 Mb sized human mini X chromosome in LA9 cells.[20] Since Southern analysis indicated that FIXACs maintain structural stability (**Figure 6c**) and FISH analysis demonstrated that the mouse centromere sequence is absent from FIXACs (**Figure 5b**; data not shown), the improved segregation efficiency in subclonal lines probably reflects more effective maintenance of centromeric chromatin and kinetochore proteins on FIXACs. Local epigenetic modifications of chromatin are thought to be important for building a foundation for the kinetochore and for maintenance of centromeric cohesion (reviewed in refs. 24,25). We are currently investigating whether chromatin composition differs between mitotically stable and unstable FIXACs in LA9 cells as this may influence their segregation potential. In hamster cell lines, FIXAC segregated with variable efficiencies. In one line (CHL15), the FIXAC segregation efficiency matched that of the human donor (**Figures 6a** and **3a**). It is likely that epigenetic effects also influence FIXAC segregation potential in hamster cells. The generation of rodent cell lines, which efficiently segregate FIXACs, suggests that FIXACs will function in transgenic animal models. The feasibility of animal studies is further supported by the stable segregation in mice of *de novo* human ACs harboring either the *GCH1* gene or the globin gene cluster.[7] The demonstration that FIXAC can be stably maintained *in vivo* without deleterious effect and provide sustained phenotypic rescue would constitute significant progress towards clinical applications.

AC based gene therapy will ultimately depend on a suitable means of AC delivery to target tissues with cultured stem or progenitor cells being the most promising vehicle. The stable segregation of truncated human and mouse chromosomal vectors in human mesenchymal cells, $CD34^+$ hematopoietic stem cells and bone marrow-derived mononuclear cells, supports the possibility that *de novo* human ACs could be delivered by this route (reviewed in refs. 22,26–29). Rapid advance in the area of stem cell biology combined with ongoing vector refinement make the goal of AC based gene therapy appear increasingly attainable.

MATERIALS AND METHODS

BAC and PAC constructs. BAC17 is VJ104-17α32, and has been described in detail (**Figure 1a**).[13] PACFIX is clone RP6-88D7 and was purchased from Children's Hospital Oakland Research Institute (Oakland, CA) and has a sequenced 158557 bp human genomic DNA insert (National Center for Biotechnology Information accession number AL033403) from Xq25-26.3 cloned into the pPAC4 vector.[30] The PACFIX insert includes the 33 kb human FIX gene, 4077 bp upstream sequence and 121780 bp of downstream sequence. A 3′ truncated copy of the *MCF2* gene is also present on the PACFIX insert.

Cell culture. HT1080 cells were grown in α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Mouse LA9 cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. CHL cells were grown in Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.

Transfection of BAC and PAC constructs into human HT1080 cells. Human ACs were generated in the HT1080 (human fibrosarcoma) cell line. BAC17 and PACFIX constructs were co-transfected into HT1080 cells using either a 1:1, 2:1 or 20:1 BAC17:PACFIX molar ratio. As a control, BAC17 was independently transfected into HT1080 cells to generate BAC17-based ACs. In each transfection, 1–2 μg of DNA was introduced using Lipofectamine Reagent (Invitrogen, Carlsbad, CA). G418 selection (300 μg/ml) (Mediatech, Herndon, VA) was applied at 48 hours post-transfection. Colonies appeared after 10–14 days and were individually expanded to generate cell lines.

FISH and immunofluorescence. Metaphase chromosomes were prepared from $G418^R$ or BS^R cell lines and analyzed using FISH to permit detection of human ACs using standard procedures. Cell lines generated from co-transfection of BAC17 and PACFIX constructs were hybridized with a chromosome 17 α-satellite (D17Z1) probe, p17H8[31], and PACFIX. Control cell lines generated from transfection of BAC17 were hybridized with the BAC vector (VJ104)[13] and p17H8 (**Figure 1b**). Probes were labeled directly with either spectrum red or spectrum green labeled deoxyuridine triphosphate using a commercial kit (Vysis, Downers Grove, IL). The primers used to amplify the consensus α-satellite probe have been described.[32] Ten micrograms of p17H8 were used to compete D17Z1 sequences in the consensus α-satellite probe (**Table 1**). The mouse minor satellite probe (**Figure 5b**) was generated by PCR using mouse LA9 genomic DNA as a template with the minor satellite primers (5′TATCCATGAGTTAC AATG3′), (5′CATTGTAACTCATTGATA3′). ≥20 metaphases were scored in each sample. Extended DNA fibers (**Figure 5d**) were prepared as before[33] and hybridized with PACFIX and p17H8 probes. Kinetochore formation on FIXACs was determined by indirect immunofluorescence using a human CENP-A antibody (gift from Manuel Valdivia[34] or purchased from AbCam, Cambridge, MA) or an anti mouse Cenp-c antibody[35] (gift from Andy Choo) on unfixed, cytospun chromosomes according to published methods.[36] A fluorescein isothiocyanate–conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used to detect CENP-A (**Figure 2e** and **f**) or Cenp-c (**Figure 5c**). Following CENP-A or Cenp-c detection, slides were hybridized with p17H8 (**Figure 2e** and **f**) or PACFIX (**Figure 5c**) probes to permit FIXAC identification. Pseudo-colored images were captured using a Leica DM5000B fluorescence microscope equipped with a Spot RTKE camera (Diagnostic Instruments, Sterling Heights, MI) and processed using Adobe Photoshop.

Mitotic stability. Lines harboring FIXACs were analyzed using FISH analysis of ≥20 chromosome spreads to determine FIXAC mitotic stability following 30–120 days of growth under non-selective conditions. In human and CHL lines, probes were PACFIX and p17H8. In L1 cells and sub-clonal lines, probes were PACFIX and minor satellite.

Microcell mediated chromosome transfer. FIXAC was transferred into rodent cells using a microcell-mediated chromosome transfer and was carried out as previously described with minor modifications.[37] Details of this method are provided as supplementary information (**Supplementary Figure S3**). FIXAC transfer into rodent cells was confirmed using FISH analysis with PACFIX and p17H8 probes. A human CotI probe (Invitrogen, Carlsbad, CA) was used to confirm that only FIXAC was transferred into recipient lines.

FIX mRNA detection by RT-PCR. Total RNA was extracted from cell lines using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was generated from 5 μg of total RNA using SuperScript First-Strand Synthesis (Invitrogen, Carlsbad, CA). Human liver RNA was purchased from Clontech (Mountain View, CA). Primers were as follows: human FIX (5′-ATGACTTCACTCG GGTTGTTG-3′), (5′-GTGAGAGGCCCTGTTAATTTTC-3′) (**Figures 4a** and **6d** and **e**); human hypoxanthine–guanine phosphoribosyltransferase (5′-CTTCCTCCTCCTGAGCAGTC-3′), (5′-CCTGACCAAGGAAAGC

AAAG-3′) (Figures 4a and 6d and e); mouse hypoxanthine–guanine phosphoribosyltransferase (5′-TTGCTGACCTGCTGGATTAC-3′), (5′-CGAG AGGTCCTTTTCACCAG-3′) (Figure 6d); Chinese hamster hypoxanthine–guanine phosphoribosyltransferase (5′- GATCCATTCCCATGA CTGTAG-3′), (5′-ATCCAACACTTCGAGAGGTC-3′) (Figure 6e). RT-PCR conditions for human and mouse cDNA were 94 °C for 2 minutes; 94 °C for 45 seconds, 58 °C for 1 minute, 72 °C for 45 seconds (35 cycles); 72 °C for 5 minutes. For hamster cDNA, RT-PCR conditions were 94 °C for 2 minutes; 94 °C for 45 seconds, 63.9 °C for 1 minute, 72 °C for 45 seconds (35 cycles); 72 °C for 5 minutes. In control RT-PCR reactions set up with each primer pair but with no cDNA added, RT-PCR products were not amplified (data not shown). Primers were intron spanning and did not amplify a product in genomic DNA (data not shown).

Northern and Southern analyses. For Northern analysis (Figure 4b), 5 µg of total RNA was loaded in each lane, electrophoresed and blotted onto Hybond N^+ membrane (GE Healthcare, Piscataway, NJ) using standard conditions. For conventional Southern analysis (Figure 3b), each lane was loaded with 10 µg of *Eco*RI digested genomic DNA. High molecular weight DNA (Figures 3c and 6c) was prepared by embedding 1.7×10^7 cells/ml (Figure 3c) or 1.7×10^6 cells/ml (Figure 6c) in agarose plugs,[13] and digested either with *Sfi*I and *Not*I (Figure 3d) or *Apa*I (Figure 6c) then separated using pulsed field gel electrophoresis on a BioRad Chef II mapper for 15 hours at 14 °C in 0.5 × Tris/borate/EDTA with an initial switch time of 0.2 seconds, final switch time of 13.0 seconds and voltage set at 6V/cm. DNA was transferred onto Hybond N^+ membrane. Filters were hybridized with deoxycytidine triphosphate–α-32^P labeled probes. Southern and Northern blots were hybridized in 7% sodium dodecyl sulfate, 0.5 mol/l NaPO4 (Figures 3b and c and 4b). The Southern blot in Figure 6c was hybridized in 7% sodium dodecyl sulfate, 0.5 mol/l NaPO4, 20% formamide. Final wash for Southern blots was 65 °C in 75 mmol/l sodium chloride/7.5 mmol/l sodium citrate/0.5% sodium dodecyl sulfate. Final wash for the Northern blot (Figure 4b) was 65°C in 15 mmol/l sodium chloride/1.5 mmol/l sodium citrate/0.5% sodium dodecyl sulfate. The FIX probe used in Figure 4b was a 1.4 kb FIX cDNA excised from plasmid PND912 (gift from Elliot Rosen, Indiana University). The FIX probe used in Figure 3b and c was a 699 bp fragment generated by PCR from human cDNA using the primers (5′-ATGCATTCTGTGGAGGCTCT-3′), (5′-GTGAGAGGCCCTGTTAATTTTC-3′). The β-actin probe (Figure 3b) was amplified from human genomic DNA using the following primer pair: (5′-CTATCCCTGTACGCCTCTGG-3′), (5′-GAAGGAAGGCTGG AAGAGTG-3′). Primers used to amplify a 0.8 kb chromosome 17 specific α-satellite probe (Figure 6c) are published.[38] Filters were exposed to a phosphorimager screen (GE Healthcare, Piscataway, NJ) and scanned using a Storm Optical Scanner (Molecular Dynamics, Sunnyvale, CA).

Real-time PCR. Semi-quantitative real-time PCR reactions were carried out in a 25 µl volume using SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) on an Applied Biosystems 7500 Real-Time PCR machine. Relative quantitation was performed using the standard curve method (Figures 3d and 4c). FIX gene copy numbers in genomic DNA (Figure 3c) were determined using the following primers: (5′-GTGAGAGGCCCTGTTAATTTTC-3′), (5′-AAGGTATCCCGG TATGTCAAC-3′). Genomic β-actin primers (Figure 3c) were (5′-CTA TCCCTGTACGCCTCTGG-3′), (5′-TGGTGGTGAAGCTGTAGCC-3′). To amplify FIX message from human cDNA (Figure 4c), intron spanning primers (5′-ATGCCAAAC CAGGTCAATTC-3′), (5′-ATGTTCACCTGC GACAACTG-3′) were used. Human β-actin control primers (Figure 4c) were also intron spanning: (5′-TTCTACAATGAGCTGCGTGTG-3′), (5′-GTACATGGCTGGGGTGTTG-3′).

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SUPPLEMENTARY MATERIAL

Figure S1. Detection of a FIX transgene in line TX10B7.
Figure S2. Detection of RT-PCR products spanning the 2.8 kb FIX mRNA.
Figure S3. Detailed method for microcell-mediated chromosome transfer of FIXACs into rodent cells.

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